Involvement of nuclear receptor RZR/RORγ in melatonin-induced HIF-1α inactivation in SGC-7901 human gastric cancer cells

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Abstract. The melatonin nuclear receptor is an orphan member of the nuclear receptor superfamily RZR/ROR, which consists of three subtypes (α, β and γ), suggesting that immunomodulatory and antitumor effects through the intracellular action of melatonin depend on nuclear signaling. In the present study, the biological mechanisms of melatonin were elucidated in association with the RZR/RORγ pathway in SGC-7901 human gastric cancer cells under hypoxia. Melatonin suppressed the activity of RZR/RORγ and SUMO-specific protease 1 (SENP1) signaling pathway, which is essential for stabilization of hypoxia-inducible factor-1α (HIF-1α) during hypoxia. Furthermore, melatonin inhibited the stability of HIF-1α in a time- and concentration-dependent manner in SGC-7901 human gastric cancer cells during hypoxia. Consistently, siRNA-RZR/RORγ effectively blocked the expression of SENP1, HIF-1α and vascular endothelial growth factor (VEGF) production in SGC-7901 cells under hypoxia, suggesting the role of nuclear receptor RZR/RORγ in melatonin-inhibited HIF-1α and VEGF accumulation. Moreover, siRNA RZR/RORγ obviously antagonized to inhibit the action of the gastric cancer cell proliferation by melatonin. Our findings suggest that melatonin suppresses HIF-1α accumulation and VEGF generation via inhibition of melatonin nuclear receptor RZR/RORγ in SGC-7901 cells under hypoxia.

Introduction

Hypoxia, which is detected in the central region of solid tumors, is a fundamental determinant of malignant tumor progression. It can also be a leading cause of angiogenesis by activation of the expression of angiogenic factors, including vascular endothelial growth factor (VEGF) (1,2). Angiogenesis, the development of new blood vessels, is essential for tumor progression since tumors require access to blood vessels for a sufficient supply of oxygen and nutrients to maintain growth and metastasis (3,4). Furthermore, an aggressive cancer phenotype that is associated with resistance to radiation therapy, chemotherapy and a poor treatment outcome can be generated as a result of the hypoxic environment within the solid tumor (5,6). A key factor in this process is hypoxia-inducible factor-1 (HIF-1), which regulates transcription of hypoxia-activated genes and consists of the HIF-1α and HIF-1β heterodimer (7). The α subunit of HIF-1α is rapidly degraded under normoxic conditions and is stabilized under hypoxia, while HIF-1β is constitutively expressed (8). Numerous studies have aimed to target HIF-1α as an anticancer strategy, and the mechanisms of several HIF-1α inhibitors have been well characterized (9,10).

Melatonin (MLT) is an indoleamine synthesized in the pineal gland and other organs, and is a major regulator in the coordination of circadian rhythms and seasonal reproduction with antioxidant, oncostatic and antiproliferative activities (11-14). It has been demonstrated that MLT exerts its complex actions by binding and activating two distinct receptor types: membrane receptors MT1 and MT2 and the nuclear receptors (15,16). MLT membrane receptors mediate their functions through a G-protein-coupled second messenger pathway and nuclear receptor signaling appears to be mediated via the transcription factor RZR/ROR, which is an orphan member of the nuclear receptor superfamily (17,18). In mammals, the MLT membrane receptors participate in the regulation of circadian and seasonal rhythms (19). The nuclear orphan receptors suggest that immunomodulatory and antitumor effects through the intracellular action of MLT depend on nuclear signaling (20,21).

Although MLT is known to inhibit the expression of HIF-1α and VEGF, its underlying mechanisms still remain unclear (22-25). Our previous studies found that MLT inhibited growth activity in murine foregastric carcinoma cells *in vivo* and *in vitro* (26,27). In the present study, we report that pharmacological concentrations of MLT have a direct anti-angiogenic effect, and we evaluated whether the nuclear orphan receptor intracellular pathways are involved in MLT-regulated hypoxia-induced HIF-1α stabilization and angiogenesis in SGC-7901 human gastric cancer cells under hypoxia.

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Materials and methods

SGC-7901 cell culture. SGC-7901 cells were purchased from the Chinese Academy of Sciences, Shanghai Institute for Biological Science. SGC-7901 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). The cells were maintained at 37˚C in a humidified incubator with 5% CO_2. $CoCl_2$ was added at a final concentration of 100 μM to mimic hypoxia. Selecting cells in good condition for the study was performed, and all experiments were repeated at least thrice. All cell culture reagents were purchased from Gibco (Invitrogen, Carlsbad, CA, USA).

Cellular proliferation and viability assay. SGC-7901 cells were plated into 96-well plates (5,000 cells/well). The cells were treated on the following day (control, 0.01, 0.1, 1 and 3 mM MLT) and cultured for 0.2, 2, 16 and 24 h. Cell viability and proliferation were assayed using a CCK-8 kit according to the manufacturer's protocol. Briefly, the cells were incubated with CCK-8 solution (10 ml/well) for 1 h before cell density was determined by measuring the absorbance at 450 nm using a Varioskan Flash (Thermo Scientific, USA).

Immunocytochemistry. SGC-7901 cells were fixed with 10% formaldehyde and embedded in paraffin. Immunocytochemical staining for RZR/RORγ was carried out. Images were captured with a Leica DM 4000B photomicroscope (magnification, x400).

VEGF ELISA assay. Cells were seeded into 60-mm diameter dishes and continually incubated for 24 h; and then different concentrations of MLT and 100 μM of $CoCl_2$ were added to the medium. After 24 h of culture in the dark, VEGF protein levels in the supernatants secreted by the cultured cancer cells were quantified by enzyme-linked immunosorbent assay (ELISA) methods. The cultured supernatants were collected and centrifuged at 12,000 rpm at 4˚C for 15 min, and then ELISA analysis was performed according to the manufacturer's instructions (VEGF-ELISA kit; R&D Systems, USA). The values of optical density (OD; A450 values) were measured at 450 nm. The total number of cells was counted by the cell-counting plate repeated at least thrice. The standard curve was determined by SPSS statistical software. The supernatants were harvested with six replicates and the experiment was performed thrice.

Western blot analysis. After treatment, the cells were harvested, washed twice with phosphate-buffered solution (PBS), and lysed by adding ice-cold lysis buffer containing 1 mM phenylmethylsulphone fluoride, pH 7.4. The protein concentration was determined using the BCA method. For western blot analysis, equal amounts of protein were separated by 12% SDS-PAGE gel electrophoresis (110 V, 1.5 h) and the membranes were blotted by wet transfer (110 V, 1.5 h, 4˚C) on polyvinyllidene fluoride (PVDF) membranes (Millipore, USA). The membranes were blocked in 5% non-fat milk solution in PBS. The membranes were then incubated with a primary antibody (dilution, 1:1,000) overnight at 4˚C. The membranes were washed with Tris-buffered saline Tween-20 (TBST) and then incubated for 1.5 h at room temperature with a secondary antibody (dilution, 1:1,000). After washing with TBST, the membranes were exposed to X-ray film (1-15 min) for visualization of the immunoreactive bands. Densitometric analysis of specific bands was performed by Quantity One (Bio-Rad, USA). The quantity of target protein was calibrated with respect to β-actin, and control value and relative intensities were obtained.

Real-time reverse transcriptase-polymerase chain reaction. Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNA was generated from 2 mg of each RNA preparation by reverse transcription using the First Strand cDNA Synthesis kit (Promega, USA). Real-time quantitative polymerase chain reaction (PCR) for the analysis of SGC-7901 expression of RZR/RORα, RZR/RORβ, RZR/RORγ, SENP1, HIF-1α, VEGF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes and cDNA was amplified using the dye SYBR-Green (Stratagene, USA) on an StepOnePlus Real-Time PCR System (Applied Biosystems). The PCR cycling conditions (40 cycles) were as follows: 30 sec at 95˚C; for 1 min at 60˚C. The fold-change in expression of each gene was calculated using the ($2^{-\Delta\Delta Ct}$) method. Product quality of PCR was monitored using post-PCR melting curve analysis at the end of the amplification cycles. The primers were as follows: RZR/RORα, 5'-GCAGGTGAAGGAGCCAGAAGG-3' and 5'-GGAACAACAGACGCCAGTAAGAAC-3'; RZR/RORβ, 5'-CCTGTATGCTGAGGTGCAGA-3' and 5'-GGTGCTAACTGCCCATTGTT-3'; RZR/RORγ, 5'-GAGGCCATTCAGTACGTGGT-3' and 5'-GCAATCTCATCCTCGGAAAA-3'; SENP1, 5'-GAGGATGGATGCTGGAGAAG-3' and 5'-TGTCTGAGGAAGGATTATCTGAG-3'; HIF1α, 5'-ACTCAGGACACAGATTTAGACTTG-3' and 5'-ATCAGTGGTGGCAGTGGTAG-3'; VEGF (A), 5'-CTTGCCTTGCTGCTCTAC-3' and 5'-ACCACTTCGTGATGATTCTG-3'; GAPDH, 5'-CCGAGAATGGGAAGCTTGTC-3' and 5'-TTCTCGTGGTTCACACCCATC-3'.

RNA interference experiments. SGC-7901 cells were transfected with siRNA for control or RZR/RORγ using PolyPlus siRNA transfection reagent (Invitrogen) according to the manufacturer's instructions and then treated with MLT for 4 h under hypoxia. In brief, siRNA (100 pmol) was mixed with transfection reagent in Opti-MEM serum-free media (Invitrogen) and incubated for 20 min at room temperature. The siRNA/transfection reagent mixture was added to the cells for 24 h. Medium was changed before the treatment with MLT under hypoxia. The sramble and RZR/RORγ siRNA sequences were as follows: NC sense, 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACACGUUCGGAGAATT-3'; RZR/RORγ-368 (A) sense, 5'-CCCGAGAUGCUGUCAAGUUTT-3' and antisense, 5'-AACUUGACAGCAUCUCGGGTT-3'; RZR/RORγ-629 (B) sense, 5'-CCUCAUAUUCCAACAACUUTT-3' and antisense, 5'-AAGUUGUUGGAAUAUGAGGTT-3'; RZR/RORγ-713 (C) sense, 5'-GGCAGAGAGAGCUUCUAUATT-3' and antisense, 5'-UAUAGAAGCUCUCUCUGCCTT-3'.

Statistical analysis. Results are presented as the mean values ± standard error of the mean (SEM). Significance between experimental values was determined by the Student's

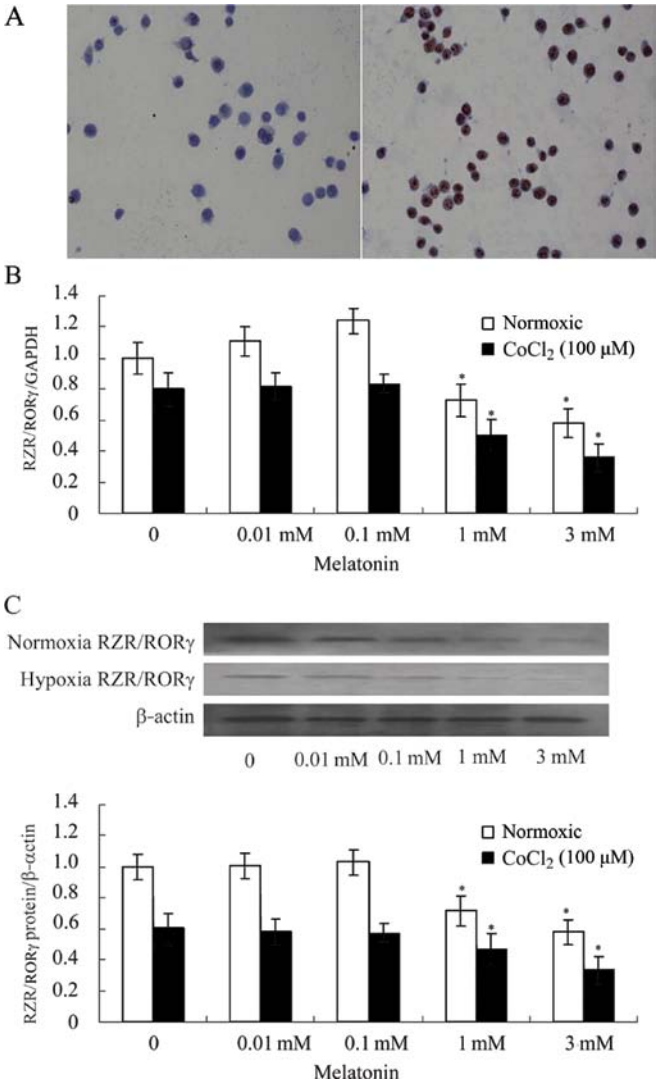

Figure 1. Cell immunohistochemical detection of the location of melatonin (MLT) nuclear receptor RZR/RORγ protein and the effect of melatonin treatment on RZR/RORγ in SGC-7901 cells. The cells were treated with MLT [0 mM (control), 0.01, 0.1, 0.1, 1 and 3 mM) for 24 h. (A) Expression and location of RZR/RORγ protein in SGC-7901 cells. (B) RZR/RORγ mRNA content. (C) RZR/RORγ protein levels. Equal loading of proteins is illustrated by β-actin bands. GAPDH was used as the gene internal control. Data are expressed as means ± SEM. *P<0.05, compared with the control cells.

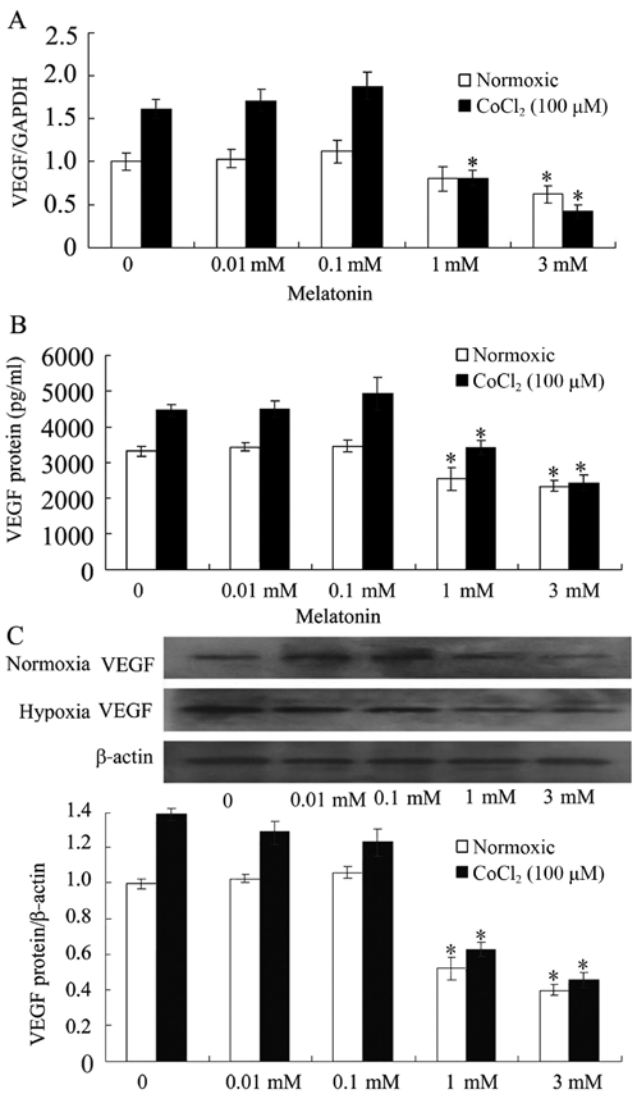

Figure 2. Melatonin (MLT) decreases vascular endothelial growth factor (VEGF) in SGC-7901 cells. Cells were treated with MLT (0 mM [control], 0.01, 0.1, 0.1, 1 and 3 mM) for 24 h. VEGF mRNA levels were assessed by reverse transcriptase-polymerase chain reaction (RT-PCR). VEGF protein concentrations in the culture media were determined. (A) VEGF mRNA levels by RT-PCR. (B) VEGF protein content in the cell supernatant by enzyme-linked immunosorbent assay. (C) VEGF protein content by western blotting. Equal loading of proteins is illustrated by β-actin bands. GAPDH was used as an internal control. Data are expressed as means ± SEM. *P<0.05, compared with the control. Data shown are representative of three independent experiments.

paired t-tests, and one-way ANOVA was used to test differences in repeated measures across experiments. Differences were considered to be statistically significant at P<0.05. Values were analyzed using the statistical package SPSS 16.0 (StatSoft, Tulsa, OK, USA).

Results

MLT nuclear receptor RZR/ROR expression in SGC-7901 cells and the effects of MLT on the mRNA and the protein expression of RZR/RORγ. The results of immunocytochemistry showed that RZR/RORγ was expressed in SGC-7901 human gastric cancer cells (Fig. 1A), and RZR/RORα and RZR/RORβ were not detected. Treatment of SGC-7901 cells with MLT (0.01, 0.1, 1 and 3 mM) for 24 h resulted in decreased expression of RZR/RORγ at the mRNA and protein levels compared with these levels in the control group under hypoxic conditions (Fig. 1B and C).

MLT decreases VEGF expression in SGC-7901 cells. To investigate the effect of pharmacological concentrations of MLT on VEGF expression in cultured cells, the levels of VEGF mRNA were detected by RT-PCR after incubation with MLT for 24 h. The results showed that 0.01 mM MLT failed to influence the basal levels and the $CoCl_2$-induced levels of VEGF mRNA. Exactly 1 mM of MLT downregulated the basal levels of VEGF mRNA. The induced levels of VEGF mRNA were significantly suppressed by 3 mM of MLT (Fig. 2A). We next assessed whether the influence on VEGF mRNA by MLT resulted in a decreased production of VEGF protein.

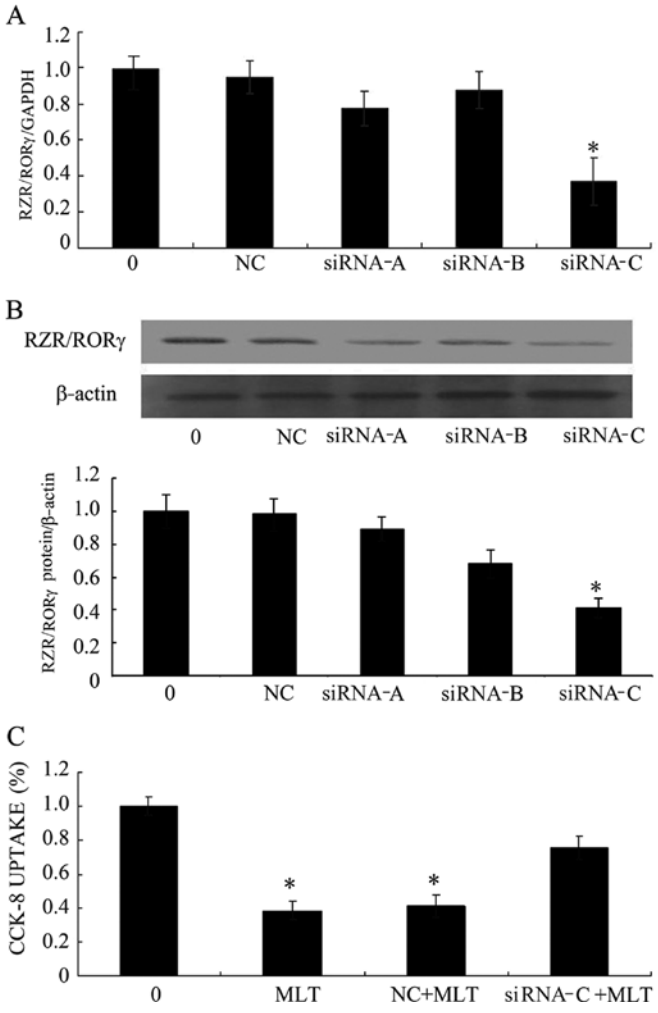

Figure 3. Decreased RZR/RORγ expression in SGC-7901 cells and the effects of melatonin (MLT) on cellular viability after application of siRNA RZR/RORγ. (A) RZR/RORγ mRNA expression by RT-PCR. (B) RZR/RORγ protein expression by western blotting. (C) Cell proliferation activity following obstruction of the RZR/RORγ signaling pathway. Equal loading of proteins is illustrated by β-actin bands. GAPDH was used as an internal control. Data are expressed as means ± SEM. *P<0.05, compared with the control. Data shown are representative of three independent experiments.

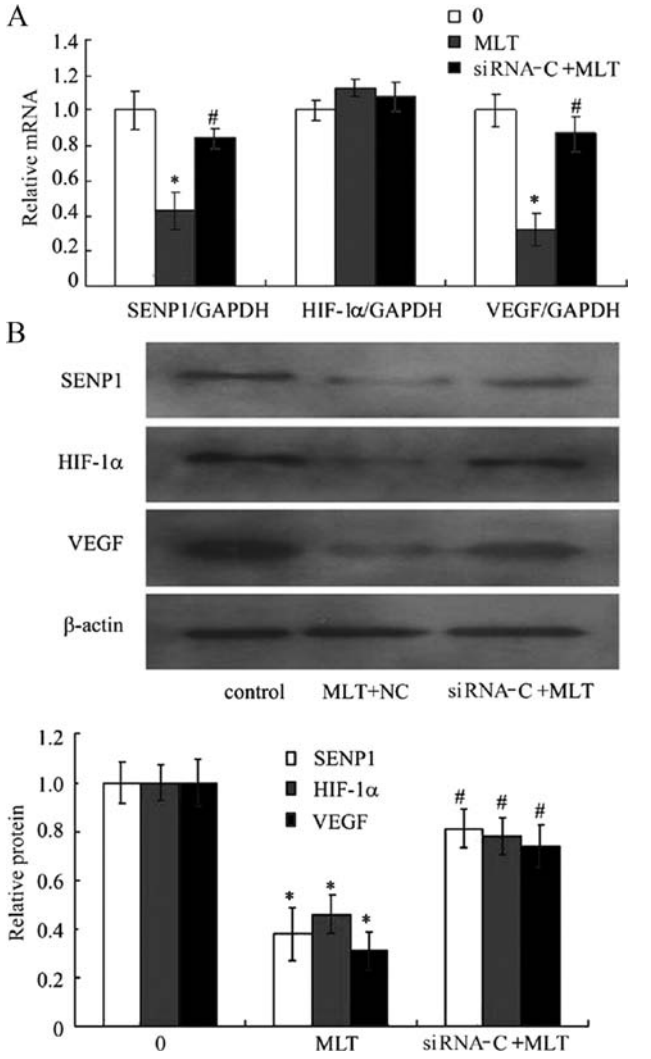

Figure 4. siRNA RZR/RORγ obviously antagonized to inhibit the downregulated basal levels of SENP1 (SUMO-specific protease 1), HIF-1α (hypoxia-inducible factor-1α), VEGF mRNA and protein in the gastric cancer cells by MTL. (A) RZR/RORγ mRNA expression by RT-PCR. (B) RZR/RORγ protein expression by western blotting. Equal loading of proteins is illustrated by β-actin bands. GAPDH was used as an internal control. Data are expressed as means ± SEM. *,#P<0.05, compared with the control. Data shown are representative of three independent experiments.

After 24 h of incubation with different concentrations of MLT, VEGF protein levels were notably decreased in the 1 and 3 mM MLT groups compared with those of the control group and the 0.01 mM MLT group in the $CoCl_2$-treated cells (Fig. 2B and C).

Decreased RZR/RORγ expression in SGC-7901 cells and the effects of MLT on cell viability following application of siRNA RZR/RORγ. Application of siRNA technology to silence RZR/RORγ decreased RZR/RORγ expression in the SGC-7901 cells (Fig. 3A and B) and obviously antagonized to inhibit gastric cancer cell proliferation by MLT (Fig. 3C).

Effects of MLT on the mRNA and the protein expression of SENP1, HIF-1α and VEGF after application of siRNA RZR/RORγ. To investigate the effect of pharmacological concentrations of MLT on RZR/RORγ, SENP1, HIF-1α and VEGF expression in cultured cells, the levels of VEGF mRNA and protein were detected by RT-PCR and western blotting after incubation with 3 mM MLT for 24 h. The results showed that 3 mM of MLT downregulated basal levels of SENP1, HIF-1α and VEGF mRNA and protein. After application of siRNA technology to silence RZR/RORγ, 3 mM MLT for 24 h obviously antagonized to inhibit the downregulated basal levels of SENP1, HIF-1α and VEGF mRNA and protein in the gastric cancer cells (Fig. 4A and B).

Discussion

Hypoxia inducible factors are transcription factors that respond to hypoxia, a pathological condition in which the body is deprived of an adequate oxygen supply (28). Notably, recent studies report that melatonin (MLT) suppressed HIF-1α activation and angiogenesis in cancer cells under hypoxia (22-24). However, the underlying mechanisms responsible for MLT inhibition of hypoxia-induced HIF-1α accumulation are not

fully understood. Thus, in the present study, we found that MLT inhibited HIF-1α activation and VEGF secretion via the MLT nuclear receptor in SGC-7901 human gastric cancer cells.

As a small lipophilic molecule, MLT easily crosses cellular membranes and exerts its biological action through nuclear signaling (29). It was proposed that the putative nuclear MLT receptor is identical to and belongs to a novel subclass of orphan nuclear receptors which suggests that immunomodulatory and antitumor effects through the intracellular action of MLT depend on nuclear signaling (17). The MLT nuclear receptors have been cloned simultaneously by two different groups and received the following names: retinoid Z receptor (RZR) and retinoid acid receptor-related orphan receptor (ROR). The RZR/ROR family consists of three subtypes (α, β and γ) and four splicing variants of the α-subtype (30). In the present study, we found that RZR/RORγ was highly expressed in SGC-7901 human gastric cancer cells while these cells did not express RZR/RORα and RZR/RORβ. Treatment of SGC-7901 cells with MLT resulted in decreased expression of RZR/RORγ in hypoxic conditions. Notably, we found that MLT treatment significantly blocked RZR/RORγ under hypoxia in SGC-7901 cells. Furthermore, RZR/RORγ siRNA obviously antagonized to inhibit the action of the gastric cancer cell SGC-7901 proliferation by MTL. Thus, MLT nuclear receptor RZR/RORγ plays an important role to inhibit the action of gastric cancer cell proliferation during hypoxia.

During normoxia, HIF1α is hydroxylated at two critical proline residues by a family of oxygen-sensitive enzymes prolyl 4-hydroxylases (PHD). Proline hydoxylated HIF1α then binds to VHL, a component of the ubiquitin E3 ligase complex consisting of Cul-2, VHL, elongin B and elongin C. Subsequently, HIF1α is ubiquitinated and degraded by the proteasome (31). Hypoxia induces nuclear translocation and SUMOylation of HIF1α, which binds to VHL in a hydroxyl proline-independent manner, leading to ubiquitination and proteasomal degradation. SUMO-specific protease 1 (SENP1), which is predominately a nuclear protein, is well-positioned to regulate the activity and stability of HIF1α in the nucleus by removing SUMO (32-34). Thus, SENP1 plays a critical role to control HIF1α stability during hypoxia. To further confirm the involvement of SGC-7901 in MLT-mediated inhibition of HIF-1a during hypoxia, we analyzed the effects of MLT on SENP1 since the SENP1-dependent stabilization of HIF-1a is known to be mediated by the SENP1 signaling pathway. In the present study, we confirmed that MLT significantly downregulated basal levels of RZR/RORγ as well as SENP1, HIF-1α and VEGF. RZR/RORγ siRNA obviously antagonized to inhibit the downregulation of basal levels of SENP1, HIF-1α and VEGF. Furthermore, the hypoxia-induced HIF-1α accumulation was significantly suppressed in the presence of MLT, consistent with previous studies.

Our data demonstrated that MLT significantly prevented hypoxia-mediated RZR/RORγ and SENP1 in SGC-7901 cells. Furthermore, RZR/RORγ siRNA transfection augmented the inhibitory effect of MLT on SENP1 and HIF-1α accumulation in SGC-7901 cells under hypoxia. Likewise, RZR/RORγ knockdown enhanced MLT-suppressed RZR/RORγ activity under hypoxia, suggesting that MLT suppresses hypoxia-induced HIF-1α inhibition via inactivation of RZR/RORγ in gastric cancer cells. There is evidence that HIF-1α can mediate VEGF secretion in cancer cells. In the present study, MLT reduced the levels of secreted VEGF protein in SGC-7901 cells, suggesting the strong inhibition of VEGF by MLT. Similarly, MLT revealed the suppressive effects on tumor angiogenesis with VEGF inhibition by targeting HIF-1α under hypoxia in prostate and colorectal cancer cells (22). Importantly, blocking RZR/RORγ activity prevented VEGF production in SGC-7901 cells, strongly demonstrating that RZR/RORγ plays a critical role in HIF-1α-mediated VEGF secretion under hypoxia.

Another group also reported that MLT suppressed tumor angiogenesis by inhibiting HIF-1α stabilization under hypoxia in HCT116 colon cancer cells (35). In contrast, we focused on the important roles of the RZR/RORγ-related pathways in MLT-induced HIF-1α inactivation and antiangiogenic activity in gastric cancer cells under hypoxia.

The present study showed that MLT inhibits RZR/RORγ, SENP1 and HIF-1α axis signaling and reduces VEGF production in SGC-7901 cells under hypoxia. Consistently, siRNA-RZR/RORγ effectively blocked the expression of SENP1, HIF-1α and VEGF production in hypoxic SGC-7901 cells. These findings suggest that MLT suppresses HIF-1α accumulation via inactivation of RZR/RORγ in hypoxic SGC-7901 cells as a potent anticancer supplement for gastric cancer therapy, which provides new ideas and approaches for the treatment of gastric cancer.

Acknowledgements

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